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RESEARCH ARTICLE

Co-expression of sperm membrane proteins CMTM2A and CMTM2B is essential for ADAM3 localization and male fertility in mice

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ABSTRACT

Chemokines are signaling proteins that are secreted to induce chemotaxis during an immunological response. However, the functions of transmembrane-type chemokine-like factor (CKLF) and the CMTM (CKLF-like MARVEL transmembrane domain containing) protein family remain to be determined. In this study, we focused on the testis-specific mouse CMTM gene cluster (*Cmtm1*, *Cmtm2a* and *Cmtm2b*) and generated CRISPR/Cas9-mediated mutant mice to examine their physiological functions. Although *Cmtm1* mutant mice were fertile, *Cmtm2a* and *Cmtm2b* double mutant mice had defects in male fertility due to impaired sperm function. We found that co-expression of sperm membrane proteins CMTM2A and CMTM2B is required for male fertility and affects the localization of the sperm membrane protein ADAM3 in regulating sperm fertilizing ability.

KEY WORDS: CKLF, CMTM gene cluster, CRISPR/Cas9, Fertilization, Testis, Zona pellucida

INTRODUCTION

Chemokines are secreted proteins that are well known for their functions in immune cell movement and lymphoid tissue development (Griffith et al., 2014). However, the functions of the transmembrane-type chemokine-like factor (CKLF) and the family of proteins CMTM1-8 (previously named CKLFSF1-8) have not been widely investigated (Han et al., 2001, 2003). Unlike chemokines, *CKLF* and *CMTM1-8* constitute gene clusters in humans and mice (Han et al., 2003; Zlotnik et al., 2006). These genes localize to three regions in human chromosomes 3, 14 and 16 and in mouse chromosomes 8, 9 and 14. Among these gene clusters, two testis-specific genes, *CMTM1* and *CMTM2*, and three ubiquitously expressed genes, *CKLF*, *CMTM3* and *CMTM4*, map to chromosome 16 in humans (Han et al., 2003; Wang et al., 2004; Shi et al., 2005). The corresponding mouse orthologs, *Cklf*, *Cmtm1*, *Cmtm2a*, *Cmtm2b*, *Cmtm3* and *Cmtm4* localize to chromosome 8. Although mouse *Cmtm1* is the single homolog of human *CMTM1*, mouse *Cmtm2a* and *Cmtm2b* are both homologs of human *CMTM2* which is highly expressed in testicular germ cells (Liu et al., 2007). Moreover, *Cmtm2a* and *Cmtm2b* are also testis-enriched genes and it is suggested that they share a similar function (Li et al., 2006). However, their physiological roles remain to be determined *in vivo*.

Recently, we established a quick and efficient system to analyze male fertility *in vivo* using CRISPR/Cas9-mediated mutant mice (Mashiko et al., 2013; Miyata et al., 2016). With this system, we found that 54 evolutionarily conserved and testis-enriched genes are not essential individually for male fertility in mice (Miyata et al., 2016). Moreover, we suggested that researchers should determine whether a gene of interest is required for male fertility *in vivo* before spending significant effort to analyze the molecular function of the gene *in vitro*.

In this study, we focused on analyzing three testis-specific genes, *Cmtm1*, *Cmtm2a* and *Cmtm2b*, in the CMTM gene cluster on mouse chromosome 8. To examine the physiological roles of these genes, we generated CRISPR/Cas9-mediated mutant mouse lines. Although *Cmtm1* mutant mice were fertile, *Cmtm2a* and *Cmtm2b* double mutant male mice were sterile. In addition, the disruption of CMTM2A and CMTM2B affected the localization of sperm membrane protein ADAM3 that is essential for male fertility (Fujihara et al., 2018; Okabe, 2018). We conclude that co-expression of sperm membrane proteins CMTM2A and CMTM2B regulates the sperm fertilizing ability and that the two proteins are new members of the ADAM3-associated protein family.

RESULTS

Testis-specific expression of *Cmtm1*, *Cmtm2a* and *Cmtm2b* in mice

In mice, the CMTM gene cluster localizes to chromosome 8 (Han et al., 2003). The gene cluster consists of six genes *Cmtm1*, *Cmtm2a*, *Cmtm2b*, *Cmtm3*, *Cmtm4* and *Cklf*. The expression of these six genes in various mouse organs was examined by RT-PCR analysis. Although *Cmtm3*, *Cmtm4* and *Cklf* were expressed ubiquitously, *Cmtm1*, *Cmtm2a* and *Cmtm2b* were exclusively expressed in mouse testes (Fig. 1A), as similarly reported in humans (Han et al., 2003). Next, the onset of *Cmtm1*, *Cmtm2a* and *Cmtm2b* expression in testes was examined by RT-PCR. All three genes were expressed 2 weeks after birth (Fig. 1B). These data show that mouse *Cmtm1*, *Cmtm2a* and *Cmtm2b* are testis-specific genes within the CMTM gene cluster and are expressed in the testes at the same stage. Mouse *Cmtm1* is a homolog of human *CMTM1* while mouse *Cmtm2a* and *Cmtm2b* are two homologs of human *CMTM2* which localizes to human chromosome 16 (Fig. 1C) (Li et al., 2006). Protein sequence similarity between CMTM1 and CMTM2 shows that four putative transmembrane regions are conserved among humans and mice (Fig. S1A-C).

Male fertility in *Cmtm1* mutant mice

To analyze the physiological role of CMTM1, we generated *Cmtm1* mutant mice by CRISPR/Cas9. A 19 bp deletion in the first exon

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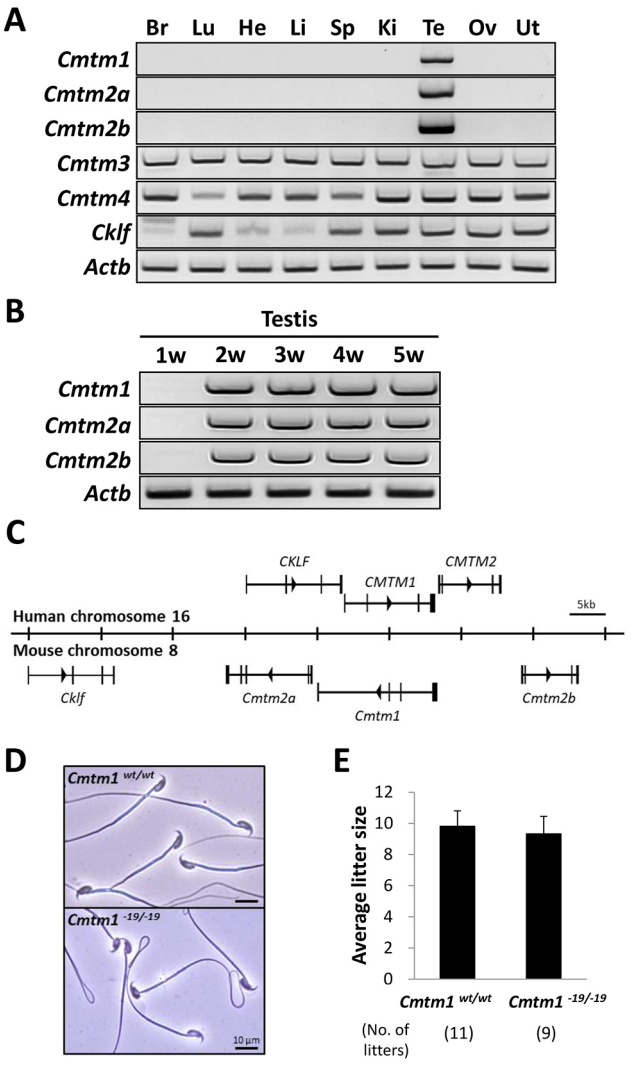

Fig. 1. Gene expression of the CMTM gene cluster and male fertility of *Cmtm1* mutant mice. (A) Testis-specific expression of the CMTM cluster by multi-tissue RT-PCR analysis. The expression of each gene was examined by RT-PCR using RNA isolated from various organs. *Cmtm1*, *Cmtm2a* and *Cmtm2b* were detected only in the mouse testis. The *Actb* gene was used as an expression control. (B) RT-PCR analysis of *Cmtm1*, *Cmtm2a*, and *Cmtm2b* in the mouse testis. All three genes were first expressed in 2-week-old testis. (C) Genomic structure of *CMTM1* and *CMTM2* in humans and mice. Evolutionarily conserved, *Cmtm2a* and *Cmtm2b* in mice are considered the homolog of human *CMTM2*. Arrowheads indicate the direction of transcription. (D) Cauda epididymal spermatozoa from wild-type and $Cmtm1^{-19/-19}$ mice. There were no differences in sperm morphology between wild-type and $Cmtm1^{-19/-19}$ mice. Scale bars: 10 µm. (E) Average litter size of wild-type and $Cmtm1^{-19/-19}$ male mice. There was no significant difference between wild-type and $Cmtm1^{-19/-19}$ male mice. $Cmtm1^{-19/-19}$ male mice were fertile.

was confirmed by PCR and direct sequencing analysis (Fig. S2A). RT-PCR analysis of the $Cmtm1^{-19/-19}$ testis showed a single band around 1.2 kb (Fig. S2B) and a 19 bp deletion from wild-type *Cmtm1* mRNA was also confirmed (Fig. S2C). The 19 bp deletion causes a frameshift mutation, leading to a premature termination codon after amino acid 132 (wild-type CMTM1 comprises 392 aa) (Fig. S2D). $Cmtm1^{-19/-19}$ mice showed no deleterious effects on testicular histology (Fig. S2E). Spermatozoa produced by $Cmtm1^{-19/-19}$ mice were motile and morphologically normal under phase-contrast microscopy (Fig. 1D). To examine male fertility, adult $Cmtm1^{-19/-19}$ males were mated with wild-type females for several months. $Cmtm1^{-19/-19}$ males were fertile, showing normal mating behavior with successful ejaculation and vaginal plug formation. The mean litter size was 9.8±1.0 in $Cmtm1^{wt/wt}$ males and 9.3±1.1 in $Cmtm1^{-19/-19}$ males (Fig. 1E). *Cmtm1* females were fertile as expected, as *Cmtm1* is a testis-specific gene (Fig. 1A). These data suggest that *Cmtm1* is not essential for male fertility in mice.

Localization of CMTM2A and CMTM2B on sperm head and male fertility of *Cmtm2a* and *Cmtm2b* double mutant mice

We prepared polyclonal antibodies to check the localization of CMTM2A and CMTM2B. Immunostaining of cauda epididymal spermatozoa was performed after the confirmation of antibody specificity (Fig. S3A). CMTM2A and CMTM2B were localized to the membrane of the sperm head when we used antibodies against CMTM2A, CMTM2B, the sperm plasma membrane protein ADAM1B and the sperm acrosomal membrane protein IZUMO1 (Fig. 2A,B and Fig. S3B). CMTM2B was detected in the sperm head using immunoblot analysis (Fig. S3C). These results confirmed that CMTM2A and CMTM2B are sperm plasma membrane proteins. As shown in Fig. 1C and Fig. S1A,C, there is high similarity of genomic localization and amino acid sequence among mouse CMTM2A and CMTM2B, and human CMTM2. There is a possibility that mouse *Cmtm2a* and *Cmtm2b* have a compensative role to each other. To examine the function of CMTM2A and CMTM2B, we produced two lines of *Cmtm2a* and *Cmtm2b* double mutant mice using CRISPR/Cas9. In one mutant line (line 1), a 2 bp deletion of *Cmtm2a* and a 444 bp insertion (via a 313 bp deletion and a 757 bp insertion event) of *Cmtm2b* was confirmed by genotyping PCR, direct sequencing (Fig. 2C, Fig. S3D and Table S1) and RT-PCR analysis (Fig. 2D). In the other mutant line (line 2), a 15 bp deletion of *Cmtm2a* and an 8 bp deletion of *Cmtm2b*, was also confirmed by genotyping PCR, direct sequencing (Figs S3E,F and S4A) and RT-PCR analysis (Fig. 2D). In line 1, a frameshift mutation occurs in both *Cmtm2a* and *Cmtm2b* ($Cmtm2a^{-2/-2}$; $Cmtm2b^{+444/+444}$). In line 2, two mutations occur in the two genes, an in-frame mutation of *Cmtm2a* caused by a five amino acid deletion (residues 13-17) and a frameshift mutation of *Cmtm2b* ($Cmtm2a^{-15/-15}$; $Cmtm2b^{-8/-8}$). Immunoblot analysis confirmed that CMTM2A and CMTM2B are disrupted in line 1 testicular germ cells (TGCs) while only CMTM2B is disrupted in line 2 TGCs (Fig. 2E). Furthermore, it was found that the smaller CMTM2A slightly remains in line 2 TGCs. Line 1 and line 2 mice showed no deleterious effects on testicular histology (Fig. S4B). Spermatozoa produced by males in both lines were morphologically normal under phase-contrast microscopy (Fig. 2F). To examine male fertility, we checked the pregnancy rate and the mean litter size of males in the two double mutant lines. The pregnancy rate was 94.4% (29/31 plugs) of female mice mated with wild-type males, 0% (0/25 plugs) of females mated with line 1 males, and 48.4% (15/31 plugs) with line 2 males (Fig. 2G). The mean (±s.d.) litter size was 8.7±3.1 for wild-type males, 0 for line 1 males and 4.0±2.7 for line 2 males (Fig. 2H). Although line 2 males were subfertile, line 1 males were completely sterile despite showing normal mating behavior with successful ejaculation and vaginal plug formation.

In vitro fertilizing ability of double mutant spermatozoa

To analyze the infertility of *Cmtm2a, Cmtm2b* double mutant males, we performed *in vitro* fertilization (IVF) assays using spermatozoa from line 1 and line 2 males mixed with cumulus-intact oocytes. Few spermatozoa collected from the cauda epididymis in line 1 mice could swim-up in sperm incubation medium, in direct contrast with

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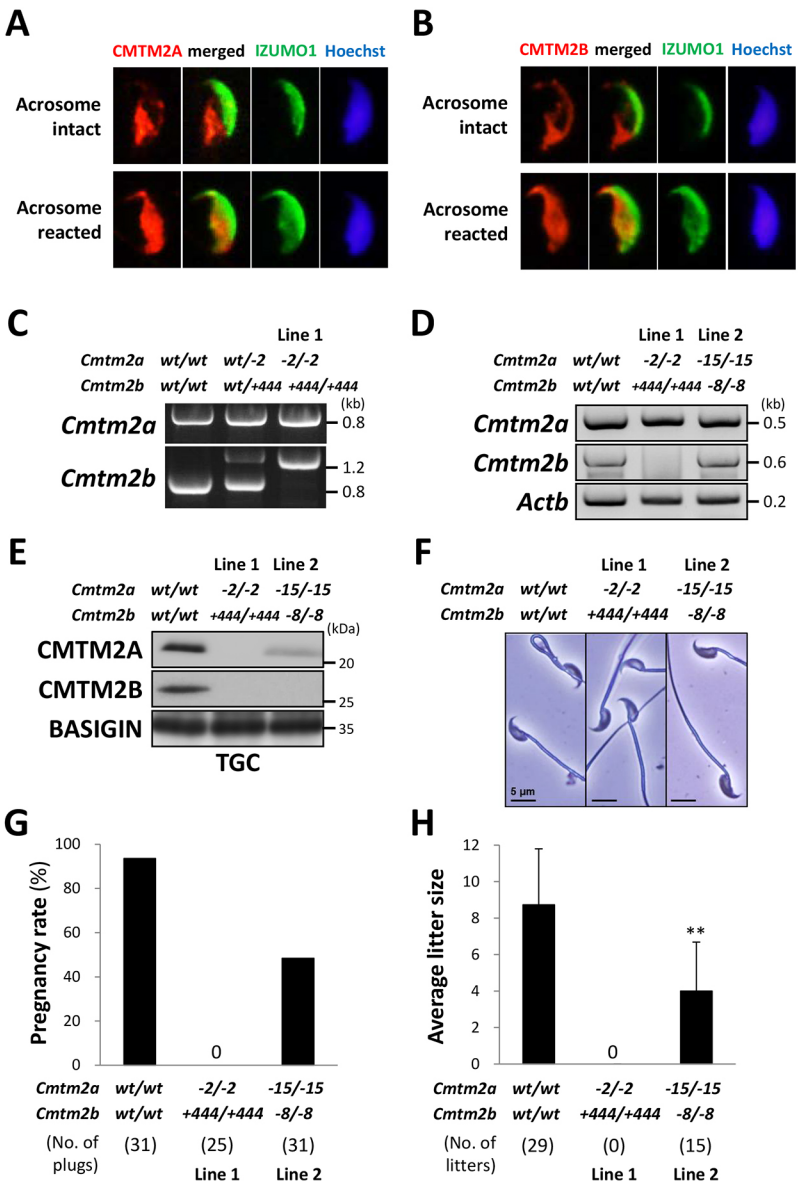

Fig. 2. Sperm membrane proteins CMTM2A/2B and male fertility of *Cmtm2a*, *Cmtm2b* double mutant mice. (A,B) Immunostaining of CMTM2A (A) and CMTM2B (B) in cauda epididymal spermatozoa. CMTM2A and CMTM2B (red signals) localize to the sperm head membrane. IZUMO1 (green signals) is a sperm acrosome membrane protein used as a marker for the acrosome reaction. Blue signals indicate sperm nuclei stained with Hoechst 33342. (C) Genotyping with PCR in *Cmtm2a*$^{-2/-2}$; *Cmtm2b*$^{+444/+444}$ mice. In wild-type mice, PCR products of *Cmtm2a* and *Cmtm2b* are represented by 834 bp and 815 bp fragments, respectively. (D) RT-PCR analysis using testis cDNA collected from wild-type, *Cmtm2a*$^{-2/-2}$; *Cmtm2b*$^{+444/+444}$ (line 1), and *Cmtm2a*$^{-15/-15}$; *Cmtm2b*$^{-8/-8}$ (line 2) mice. In wild-type testis, PCR products of *Cmtm2a* and *Cmtm2b* are represented by 510 bp and 633 bp fragments, respectively. *Cmtm2b* mRNA is not detected in line 1 testes. (E) Immunoblot analysis using testicular germ cell (TGC) lysates collected from wild-type, line 1 and line 2 mice. Both CMTM2A and CMTM2B were disrupted in line 1 testes, although CMTM2B was disrupted in line 2 testes. (F) Cauda epididymal spermatozoa from wild-type, line 1 and line 2 mice. Scale bars: 5 µm. (G) Pregnancy rates of wild-type female mice mated with wild-type, line 1 and line 2 male mice. Pregnancy rate is the success rate from natural matings (number of pregnancies per plug). The average pregnancy rate of females coupled with wild-type male mice was 93.5% (29/31). The rate of pregnancy of female mice coupled with line 1 male mice was 0% (0/25) and the rate of line 2 male mice was 48.4% (15/31). (H) Average litter size of wild-type, line 1, and line 2 male mice. Average litter size was measured by the number of pups in each delivery. Line 1 male mice were completely infertile. Although the mean(±s.d.) litter size sired by wild-type male mice was 8.7±3.1, the average for line 2 male mice was 4.0±2.7. ***P*<0.001.

line 2 spermatozoa that could. The major cause of sterility in line 1 mice was due to immotile epididymal spermatozoa. Line 2 spermatozoa could fertilize with eggs as effectively as wild-type spermatozoa [92.4±9.3% (330/357 eggs) and 98.4±2.8% (121/123 eggs) by wild-type and line 2, respectively] (Fig. 3A). To examine the subfertility of line 2 mice, we checked the sperm zona pellucida (ZP)-binding ability of the spermatozoa mixed with cumulus-free oocytes *in vitro*. Line 2 spermatozoa had a reduced ability to bind to the ZP compared with the wild-type spermatozoa (45.8±11.2 and 3.9±2.2 spermatozoa per egg by wild-type and line 2 spermatozoa, respectively; $P<0.001$) (Fig. 3B,C). These results indicated that spermatozoa produced by line 1 were immotile, and the spermatozoa of line 2 had an impaired ZP-binding ability.

Immunoblot analysis of TGC and spermatozoa in double mutant mice

The impaired ZP-binding phenotype in line 2 mice is shared among mice lacking sperm membrane protein ADAM3 (Fujihara et al., 2018). Thus, we performed an immunoblot analysis of ADAM3-associated proteins in the *Cmtm2a, Cmtm2b* double mutant mice. While ADAM3 was not detectable in line 1 spermatozoa, there were no differences between wild-type, line 1 and line 2 TGCs (Fig. 3D,E). In contrast, ADAM3 was readily detected in line 2 spermatozoa (Fig. 3E). Moreover, in line 2, CMTM2A remained in TGCs (Fig. 2E) but was not detected in spermatozoa (Fig. 3E). Although both CMTM2A and CMTM2B were absent in line 1 and 2 spermatozoa, ADAM3 was only missing in line 1 spermatozoa.

CMTM2A and CMTM2B in *Adam3* KO and *Adam3-Flag* transgenic rescue mice

As shown in Fig. 3, there is a possibility that CMTM2A, CMTM2B and ADAM3 interact in mouse testis and spermatozoa. We checked CMTM2A and CMTM2B (CMTM2A/2B) levels in *Adam3* knockout (KO) mice by immunoblot analysis after generating *Adam3* KO mice (Fig. S4C). CMTM2B disappeared from spermatozoa in *Adam3* KO mice, while CMTM2A remained (Fig. 4A). We reported previously that KO spermatozoa from a TGC-specific GPI-anchored protein encoded by *Ly6k* possess the same phenotype in which the spermatozoa lose their fertilizing ability, but ADAM3 remains (Fujihara et al., 2014), which is similar

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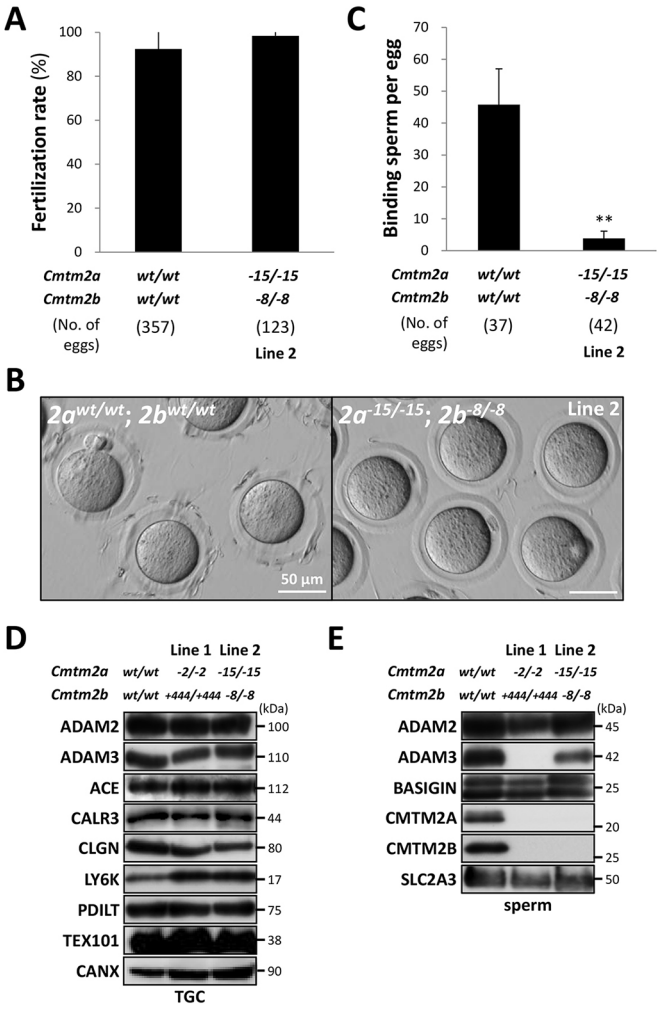

Fig. 3. *In vitro* fertilizing ability and immunoblot analysis of *Cmtm2a, Cmtm2b* double mutant mice. (A) *In vitro* fertilization rates using wild-type and *Cmtm2a*$^{-15/-15}$; *Cmtm2b*$^{-8/-8}$ (line 2) spermatozoa. Average fertilization rates of wild-type and line 2 spermatozoa were 92.4±9.3% (330/357 eggs) and 98.6±2.8% (121/123 eggs), respectively. (B) Observation of ZP-binding in wild-type and line 2 spermatozoa. Line 2 spermatozoa have an impaired ZP-binding ability *in vitro*. Scale bars: 50 µm. (C) Average number of ZP-binding spermatozoa *in vitro*. The number of ZP-binding spermatozoa in line 2 mice (3.9±2.2 spermatozoa; mean±s.d.) was significantly reduced compared with that of wild-type mice (45.8±11.2 spermatozoa). ***P*<0.001, Student's *t*-test. (D) Immunoblot analysis using TGC lysates collected from wild-type, line 1 and line 2 mice. (E) Immunoblot analysis using sperm lysates collected from wild-type, line 1 and line 2 mice.

to the results in line 2 mice. However, there were no differences in CMTM2A or CMTM2B levels in the *Ly6k* KO mice (Fig. 4B). These data imply a relationship between CMTM2A/2B and ADAM3 but no relationship with LY6K.

Next, to analyze the interaction of CMTM2A/2B with ADAM3 by immunoprecipitation (IP), we generated *Adam3-Flag* transgenic (Tg) mice because no anti-ADAM3 antibodies are available for IP analysis. The transgene utilized a truncated form of ADAM3 (736 aa), which removed the intracellular region of ADAM3 (86 aa from the C-terminus), corresponding to a region of low conservation among other species (Table S2). To confirm that the *Adam3-Flag* transgene was functional, we performed a rescue experiment by generating *Adam3-Flag* Tg mice on an *Adam3* KO background (Fig. 4C). Our results showed that the infertile phenotype of *Adam3* KO males was rescued by the transgene (average litter size of 7.0±0.9; mean±s.d.) (Fig. 4D). Expression of the transgene in these mice was further examined by immunoblot analysis. Although ADAM3 and FLAG expression from the transgene was detected in the TGCs of the *Adam3* KO-Tg mice, both could not be detected in the spermatozoa (Fig. 4E). Also, CMTM2B could not be detected in the spermatozoa produced by the *Adam3* KO-Tg mice (Fig. 4E). To check the interaction of ADAM3 with CMTM2A/2B in TGCs and spermatozoa, we performed IP analysis using FLAG antibody. ADAM3 in TGCs could be pulled down efficiently as shown by the immunoblot analysis using ADAM3 and FLAG antibodies (Fig. S4D), although IP of sperm lysates did not work as well as expected (Fig. S4E). However, CMTM2A/2B, LY6K and PDILT could not be detected in the FLAG immunoprecipitates (Fig. S4D,E). Unfortunately, these IP-immunoblot analyses using *Adam3-Flag* Tg rescue mice could not confirm the interaction between ADAM3-associated proteins (LY6K and PDILT), as reported previously (Tokuhiro et al., 2012; Fujihara et al., 2014). Under these rescue conditions, CMTM2A/2B could not interact with ADAM3 in the TGCs and spermatozoa. Therefore, we could not clarify whether CMTM2A and CMTM2B directly affect the correct localization of ADAM3 in spermatozoa.

DISCUSSION

Chemokines are a well-known family of cytokines used to recruit from immune cells (Nagarsheth et al., 2017). The transmembrane-type chemokine like factor (CKLF) and the protein family CMTM (CKLF-like MARVEL transmembrane domain containing) 1-8 constitute gene clusters in humans and mice. In this cluster, there are testis-specific expressed genes; *CMTM1* and *CMTM2* in humans and *Cmtm1*, *Cmtm2a*, and *Cmtm2b* in mice (Wang et al., 2004; Shi et al., 2005; Li et al., 2006). However, their physiological functions remained to be determined *in vivo*.

In this paper, we generated mutant mice of three testis-specific CMTM genes (*Cmtm1*, *Cmtm2a* and *Cmtm2b*) using CRISPR/Cas9. When taking human and mouse genomic conservation data into account, we disrupted these genes using two approaches: a single mutation of *Cmtm1* and double mutations of *Cmtm2a* and *Cmtm2b*. We found that although *Cmtm1* mutant mice were fertile, *Cmtm2a* and *Cmtm2b* double mutant mice were male infertile due to impaired sperm function. Immunostaining of spermatozoa indicated that CMTM2A and CMTM2B localized to the plasma membrane on the sperm head. Two mouse lines of *Cmtm2a* and *Cmtm2b* double mutations, *Cmtm2a*$^{-2/-2}$; *Cmtm2b*$^{+444/+444}$ (line 1) and *Cmtm2a*$^{-15/-15}$; *Cmtm2b*$^{-8/-8}$ (line 2) were produced and it was found that line 1 was completely infertile and line 2 was subfertile. The difference between the two phenotypes is attributed to the genetic mutations induced by CRISPR/Cas9. Line 1 was a complete knockout of *Cmtm2a* and *Cmtm2b* while line 2 had an in-frame mutation (15 bp deletion) of *Cmtm2a* and a frameshift mutation (8 bp deletion) of *Cmtm2b*. The results indicate that *Cmtm2a* and *Cmtm2b* are required for male fertility in mice.

Although the two mouse lines had different mutations, they produced normal-looking spermatozoa, but these epididymal spermatozoa showed different phenotypes: immotility and impaired ZP-binding. The impaired ZP-binding phenotype of line 2 mice is a shared phenotype among mice lacking ADAM3 (Fujihara et al., 2018). The sperm membrane protein ADAM3 is thought to play a pivotal role in sperm-ZP binding and sperm migration through the uterotubal junction (UTJ) (Shamsadin et al., 1999; Yamaguchi et al., 2009). Currently, more than 10 proteins (ACE, ADAM1A, ADAM2, CALR3, CLGN, PDILT, PMIS2,

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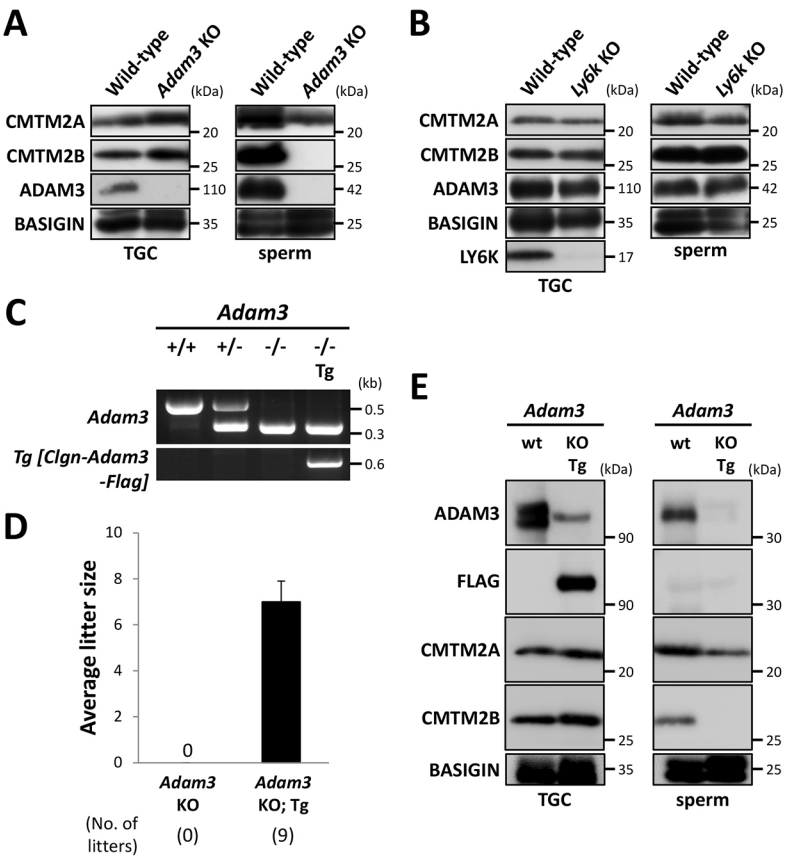

Fig. 4. CMTM2A/2B in *Adam3* and *Ly6k* KO mice and the interaction of CMTM2A/2B and ADAM3. (A) Immunoblot analysis of CMTM2A and CMTM2B using *Adam3* KO mice. Although CMTM2A and CMTM2B remained in *Adam3* KO testes, CMTM2B disappeared from *Adam3* KO spermatozoa. (B) Immunoblot analysis of CMTM2A and CMTM2B using *Ly6k* KO mice. CMTM2A and CMTM2B were detected in *Ly6k* KO testes and spermatozoa. (C) Genotyping with PCR in *Adam3-Flag* transgenic (Tg) rescue mice. A 522 bp band representing the wild-type allele, a 358 bp band representing the knockout allele, and a 614 bp band representing the Tg allele were amplified by PCR. (D) Average litter size of *Adam3-Flag* Tg rescued *Adam3* KO male mice. *Adam3-Flag* transgene restored male infertility in *Adam3* KO mice. The average litter size of *Adam3-Flag* Tg rescued *Adam3* KO male mice was 7.0±0.9 (mean±s.d.). (E) Immunoblot analysis of TGC and sperm lysates collected from wild-type and *Adam3-Flag* Tg rescued *Adam3* KO mice.

PRSS37, RNASE10, TEX101 and TPST2) have been described to affect the ADAM3 protein and/or its localization in the spermatozoa (Fujihara et al., 2018). Although ADAM3 remains in line 2 spermatozoa, it is not present in line 1 spermatozoa. This finding suggests that testicular CMTM2A may affect the correct localization of ADAM3 onto spermatozoa. Impaired ZP-binding in line 2 may be caused by abnormalities in sperm protein(s) other than ADAM3. Further experiments may be required to examine the cause of immotile spermatozoa and the disappearance of ADAM3 in line 1 mice. Therefore, our *Cmtm2a, Cmtm2b* double mutant mice may prove useful in elucidating the physiological function of human CMTM2. CMTM2B was absent in *Adam3* KO spermatozoa, while CMTM2A remained. Moreover, we recently identified TGC-specific GPI-anchored protein LY6K which is essential for sperm migration through the UTJ and ZP-binding (Fujihara et al., 2014). We showed here that *Ly6k* KO spermatozoa retained not only ADAM3 but also CMTM2A/2B. These results suggest that LY6K interacts with other unknown factor(s), but not CMTM2A/2B, to regulate sperm fertilizing ability.

To examine the interaction of ADAM3 and CMTM2A/2B, we generated Tg rescue mice using an *Adam3-Flag* transgene. The transgene used was a truncated form of ADAM3 (736 aa) which removed the protein's intracellular region (86 aa from the C-terminus). The transgene of truncated ADAM3 was functional and could rescue the male infertility of *Adam3* KO mice. However, in the Tg mouse spermatozoa, ADAM3-FLAG expression could not be detected by immunoblot analysis. Previously, we reported a similar result in the full-length *Adam3* Tg rescue mice (Yamaguchi et al., 2009). The transgenic expression of ADAM3 showed very weak signals in the testis and spermatozoa in *Adam3* Tg#47 mice. These results indicated that the amount of ADAM3 protein required for sperm fertilization is much less than the levels of ADAM3 found in wild-type spermatozoa, and that the intracellular region (residues 737-822) was not required for the sperm fertilizing ability. Since ADAM3 and FLAG were expressed in the TGCs of *Adam3-Flag* Tg mice, IP-immunoblot analysis was performed using FLAG antibody. Even though ADAM3 and FLAG were immunoprecipitated efficiently, they did not interact with either CMTM2A/2B or LY6K and PDILT as control proteins. Although we could not clarify the interaction of CMTM2A/2B and ADAM3 in our experimental conditions, KO mouse experiments indicated that CMTM2A/2B are new members of the identified group of proteins involved in ADAM3 regulation. Unfortunately, our antibodies against CMTM2A/2B and ADAM3 did not work for IP analysis. Further investigations will be required to clarify the interaction of CMTM2A/2B and ADAM3. Our findings support a potential role of human CMTM2 in sperm function and could be used to develop infertility treatments as well as contraceptives.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University (H25-02-0 and H30-01-0; 21 June 2013 and 4 July 2018, respectively). Mice were maintained under a 12 h:12 h light:dark cycle (lights on from 08:00 to 20:00). Wild-type mice were purchased from CLEA Japan (Tokyo, Japan) and Japan SLC (Shizuoka, Japan).

In this study, we generated genetically modified mouse lines, *Cmtm1* (B6D2-*Cmtm1*<em1Osb>); RBRC10119, *Cmtm2a*$^{-2/-2}$; *Cmtm2b*$^{+444/+444}$ (Line 1) (B6D2-*Cmtm2a*<em2Osb>*Cmtm2b*<em2Osb>); RBRC09947, *Cmtm2a*$^{-15/-15}$; *2b*$^{-8/-8}$ (line 2) (B6D2-*Cmtm2a*<em1Osb>*Cmtm2b*<em1Osb>); RBRC09946, *Adam3* knockout mice [STOCK-*Adam3*<tm1(KOMP)Osb>/11B]; RBRC05866, and *Adam3-Flag*

transgenic mice [STOCK-Tg(*Clgn-Adam3Flag*)1Osb]; RBRC09960. These were deposited to the RIKEN BioResource Research Center (http://mus.brc.riken.jp/en/) and the Center for Animal Resources and Development (CARD), Kumamoto University (http://card.medic.kumamoto-u.ac.jp/card/english/). The *Ly6k* knockout mouse line (STOCK-*Ly6k*<tm1Osb>) was described previously (Fujihara et al., 2014).

RT-PCR analysis

Mouse cDNA was prepared from multiple adult tissues and from 1- to 5-week-old testes of ICR mice (Fujihara et al., 2010). To confirm the mutations in *Cmtm1*$^{-19/-19}$, *Cmtm2a*$^{-2/-2}$; *Cmtm2b*$^{+444/+444}$ and *Cmtm2a*$^{-15/-15}$; *Cmtm2b*$^{-8/-8}$ mice, RT-PCR was performed using RNA from each mouse testis. The amplification conditions were 1 min at 94°C, followed by 30 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s, with a final 7 min extension at 72°C. The primers used are listed in Table S3.

Antibodies

Rabbit anti-mouse CMTM2A and CMTM2B polyclonal antisera were produced by immunization with mouse CMTM2A (residues 6-24: KFPFRPRGGQPREDTTPKR; residues 157-169: QFQHFRGLRLRKW) and CMTM2B (residues 192-210: ERQRELAEKAKRESMDPGW) polypeptides, respectively. The monoclonal antibodies used here were as described previously: TES101 for TEX101, 1D5 for ACE, KS64-10 for SLC2A3, KS64-125 for IZUMO1 and KS107-158 for ADAM1B (Ikawa et al., 2011; Fujihara et al., 2013b). Other antibodies were purchased from Sigma-Aldrich (M2 for FLAG), Santa Cruz Biotechnology (sc-9757 for BASIGIN), and Merck Millipore (7C1 for ADAM3 and 9D for ADAM2). Rabbit antisera against CLGN, CALR3 and PDILT were as described previously (Tokuhiro et al., 2012). Dilutions used were 1:200 to 1:300 for immunostaining and 1:500 to 1:1000 for immunoblot analysis.

Immunostaining

Immunostaining was performed as described previously (Fujihara et al., 2012; Muto et al., 2016).

Immunoblot

Immunoblot analysis was performed as described previously (Fujihara et al., 2017). Briefly, testicular germ cells were collected from the seminiferous tubules of testes. Sperm samples were collected from the cauda epididymis and vas deferens. Sperm head-tail separation using centrifugation was performed as described previously (Miyata et al., 2015). These samples were homogenized in lysis buffer containing 1% Triton X-100 and 1% protease inhibitor (Nacalai Tesque, Kyoto, Japan) and then were centrifuged (10,000 ***g*** for 20 min at 4°C), and the supernatants were collected. Protein lysates were separated by SDS-PAGE under reducing condition and transferred to PVDF membranes (Merck Millipore). After blocking, blots were incubated with primary antibodies overnight at 4°C, and then incubated with secondary antibodies conjugated with horseradish-peroxidase. The detection was performed using an ECL plus western blotting detection kit (GE Healthcare) and Chemi-Lumi One Ultra (Nacalai Tesque).

Generation of *Cmtm1*, *Cmtm2a* and *Cmtm2b* mutant mice with CRISPR/Cas9

Cmtm1, *Cmtm2a* and *Cmtm2b* mutant mice were produced by microinjection of pX330 plasmid (https://www.addgene.org/42230/) into mouse embryos as described previously (Mashiko et al., 2013; Fujihara and Ikawa, 2014). A search for sgRNA and off-target sequences was performed using CRISPRdirect software (https://crispr.dbcls.jp/) (Naito et al., 2015). After the validation of EGFP expression assay (Fujihara and Ikawa, 2014), the sgRNA sequence used for microinjection were: 5′-AG-GTCGTAAAGTTAAGCGTC-3′ for the first exon of *Cmtm1*, 5′-TTTCC-ATTTCGGCCCAGAGG-3′ for the first exon of *Cmtm2a*, and 5′-GATGAAAGAAGAGGGTTCAA-3′ for the first exon of *Cmtm2b*. The sgRNAs for *Cmtm2a* and *Cmtm2b* were co-injected into the pronuclei of fertilized eggs. The 2-cell-stage embryos were transferred into the oviducts of pseudopregnant ICR females the next day. *Cmtm1* mutant mice had a 19 bp deletion (5′-GCGTGAGTCCAGACGCTTA-3′) in the first exon. Two lines of *Cmtm2a* and *Cmtm2b* double mutant mice were generated; line 1: a 2 bp deletion (5′-AG-3′) in the first exon of *Cmtm2a* and a total of a 444 bp insertion mutation (313 bp deletion and 757 bp insertion) around the first and second exons of *Cmtm2b*; and line 2: a 15 bp deletion (5′-GAGGTCAGCCCAGAG-3′) in the first exon of *Cmtm2a* and an 8 bp deletion (5′-TTCAAAGG-3′) in the first exon of *Cmtm2b*. The primers used are listed in Table S3. Detailed genotype information of mutant mouse lines is shown in Figs S2-S4.

Male fertility test

Sexually mature mutant male mice were caged with 2-month-old B6D2F1 or mutant females for several months, and the number of pups in each cage was counted within a week of birth. Pregnancy rates are presented as the success rate for getting pregnant from natural matings. Average litter sizes are presented as the number of total pups born divided by the number of litters for each genotype.

Testis histology and sperm morphology

After breeding studies, males were euthanized by cervical dislocation following anesthesia. Testes were fixed in 4% paraformaldehyde in PBS and were processed for paraffin embedding. Paraffin sections were cut at 5 µm and stained with periodic acid-Schiff (PAS) and then counterstained with Mayer hematoxylin solution (Wako). The cauda epididymal spermatozoa were dispersed in PBS and then the sperm morphology was observed under a phase-contrast microscope (BX50, Olympus).

Sperm ZP-binding assay

Sperm ZP-binding assay was performed as described previously (Yamaguchi et al., 2006). Briefly, 30 min after mixing with 2-h-incubated spermatozoa, eggs were fixed with 0.25% glutaraldehyde. The bound spermatozoa were observed with an Olympus IX70 fluorescence microscope after Hoechst 33258 staining.

In vitro fertilization

In vitro fertilization using mouse spermatozoa was performed as described previously (Tokuhiro et al., 2012).

Generation of *Adam3* knockout mice with IKMC vector

The mouse *Adam3* gene targeting vector (DPGS00107_A_C12) was from the International Knockout Mouse Consortium (IKMC) (Skarnes et al., 2011). After linearization with *Asi*SI digestion, the targeting vector was electroporated into EGR-G01 (129S2×[*CAG/Acr-Egfp*]C57BL/6NCr) embryonic stem (ES) cells (Fujihara et al., 2013a), and colonies were screened. To disrupt the *Adam3* gene, exons 12-16 were replaced with an IRES:*lacZ* trapping cassette and a floxed promoter-driven neo cassette, and a diphtheria-toxin-A-chain (DTA) expression cassette was used for negative selection. After G418 selection, 1 of 96 drug-resistant clones had a homologous recombination event after PCR analysis. The mutant ES cell clones were injected into 8-cell-stage ICR embryos, and the chimeric blastocysts were transferred into the uterine horns of pseudopregnant ICR females the next day. The obtained chimeric males were mated with B6D2F1 females for germ-line transmission. Offsprings from heterozygous intercrosses were genotyped by PCR. Both a 522 bp band as the wild-type allele and a 358 bp band as the knockout (KO) allele were amplified by PCR. The primers used are listed in Table S3. The phenotype of *Adam3* KO mice was reported previously (Shamsadin et al., 1999; Yamaguchi et al., 2009).

Generation of *Adam3-Flag* transgenic rescue mice

A transgenic (Tg) rescue mouse line was produced by injecting the transgene into *in vitro* fertilized eggs from *Adam3* KO mice. The cDNA encoding the mouse *Adam3* gene was amplified by PCR using wild-type testis cDNA as a template. The *Adam3* cDNA was further modified by the removal of 86 AAs (residues 737-822) from the C-terminus of ADAM3. The *Xba*I and *Eco*RV sites included in the primers were used to introduce the modified *Adam3* cDNA into the pClgn1.1 vector, a pBluescript II SK (+) vector containing the mouse *Clgn* promoter and rabbit *globin* polyA signal (Ikawa et al., 2001). Moreover, the FLAG sequence (DYKDDDK) was

inserted into the C-terminus of the modified *Adam3* cDNA. The *Kpn*I- and *Sac*I-digested transgene fragment (3.2 kb) was purified by gel electrophoresis. The digested transgene fragment was microinjected into the pronuclei of fertilized eggs, and an *Adam3-Flag* Tg rescue mouse line [STOCK-Tg(*Clgn-Adam3Flag*)1Osb] was established. A 614 bp band as the Tg allele was amplified by PCR. The primers used are listed in Table S3.

Immunoprecipitation

Immunoprecipitation was performed as described previously (Tokuhiro et al., 2012). Briefly, the protein lysates (supernatants) were incubated with antibody-conjugated Dynabeads Protein G (Life Technologies) for 60 min at 4°C. The protein complexes were then washed three times with TBS buffer containing 40 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol before SDS-PAGE and immunoblot analysis.

Statistical analysis

Statistical analyses were performed using Student's *t*-test inserted into Microsoft Excel after the data were tested for normality of distribution. Differences were considered significant at $P<0.001$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Y.F., M.I.; Validation: Y.F., M.I.; Formal analysis: Y.F., M.I.; Investigation: Y.F., A.O., K.K., T.L., M.I.; Resources: Y.F., M.I.; Data curation: Y.F., A.O., K.K., T.L., M.I.; Writing - original draft: Y.F., M.I.; Writing - review & editing: Y.F., M.I.; Visualization: Y.F., A.O., K.K., T.L.; Supervision: M.I.; Project administration: Y.F., M.I.; Funding acquisition: Y.F., M.I.

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Data availability

All mutant mouse strains were deposited in the RIKEN BioResource Research Center (http://mus.brc.riken.jp/en/) and the Center for Animal Resources and Development (CARD), Kumamoto University (http://card.medic.kumamoto-u.ac.jp/card/english/).

Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.221481.supplemental

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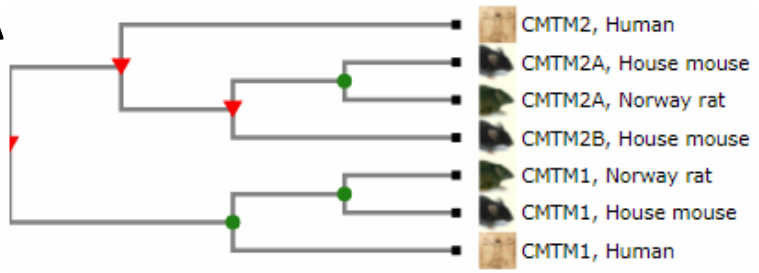
A



B

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H. sapiens_CMTM1     0 -------------------------------------------------- 0

M. musculus_CMTM1  101 SRQQSFIRATPIRQQSFIRAIPSRQRPSVQAIPSSQ--RPLVHVIPSSQR 148
H. sapiens_CMTM1     1 -------------------MDPEHAKPESSEAPSGNLKQPETAAALASSG 31

M. musculus_CMTM1  149 PSVRATPSSQRPSVRATPSSQRPSVRAVPF-SQRPTRSLQSVYRDRIADR 197
H. sapiens_CMTM1    32 SVVSSVPKAQRNISAKTAPRKHPAVSIRSAQSAAAARPQGSEGTAPSRKA 81

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H. sapiens_CMTM1    82 TTRPPPKPTLPPPTPSAHTESKLLNEMAIKERVEGRAKVPYKFRDSLKRF 131

M. musculus_CMTM1  248 CCSANGMLKVLRMCITATSVFCFVIGGAQELFVVIMIQEICIVLFFIIIY 297
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H. sapiens_CMTM1    232 LCLTAVIVCCIDAFVVTTKMRTNLKRFLGVEVERKLSPAKDAYPETGPDA 281

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H. sapiens_CMTM1    282 PQRPA                                          286
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C

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M. musculus_CMTM2B    1 ---------------------------------------------MAA 3
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M. musculus_CMTM2B    4 PAPRARTGGKKKDERRGFKGIKWEFRDSNKDFWAQGHAECKSLIMILLIA 53
H. sapiens_CMTM2     51 KPQKAVQPKHEVGTRRGCRRYRWELKDSNKEFWLLGHAEIKIRSLGCLIA 100

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M. musculus_CMTM2B   54 AMVCFQRVATHPIVILLLTMELSICAFFFFLYSLAINRYIPFVFWPMMDL 103
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M. musculus_CMTM2A  159 QHFRGLRLRKW------------------------------------------ 169
M. musculus_CMTM2B  154 RQFKSRKLRKFILLTPDRKGKKQDPKLLLMLAAKEDEEERQRELAEKAKR 203
H. sapiens_CMTM2    201 NHFRGKKAKKHMLVPPPGKFKGPQQGKGPEPAKPPEPGKPPGPAKGKK-- 248

M. musculus_CMTM2A  169 -------                                        169
M. musculus_CMTM2B  204 ESMDPGW                                        210
H. sapiens_CMTM2    248 -------                                        248
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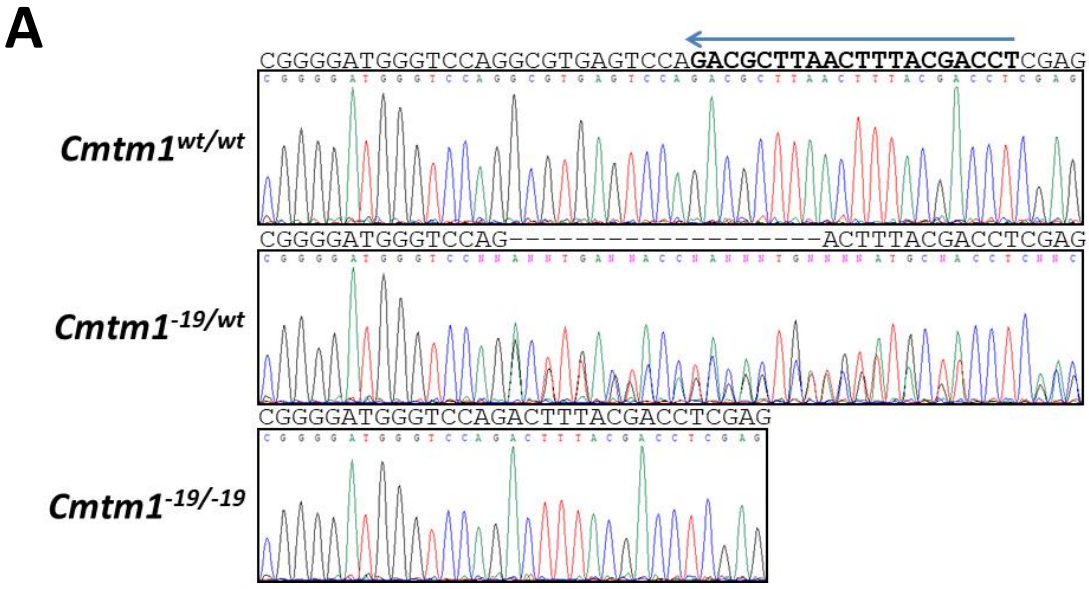
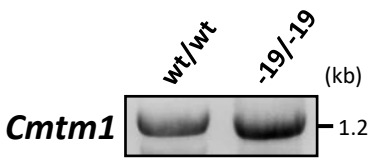
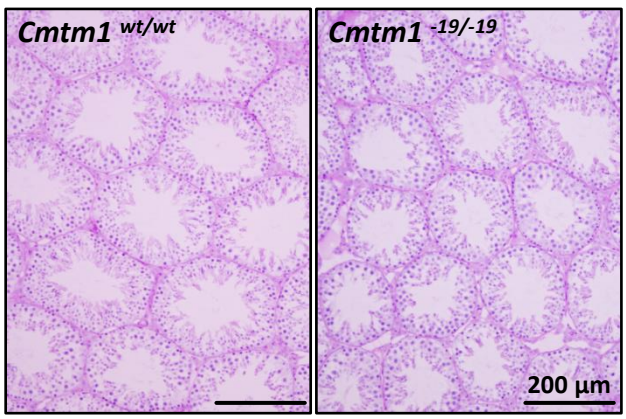
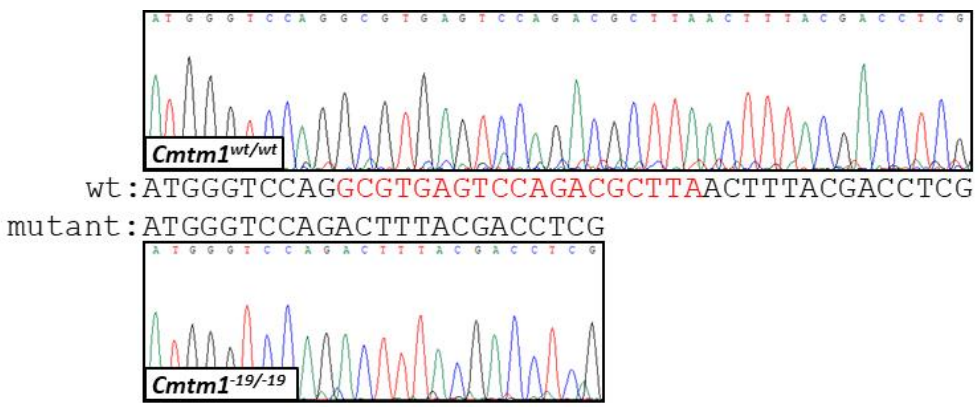
Figure S1. Amino acid sequence similarity of CMTM1 and CMTM2 in mice and humans.

(A) Gene tree of CMTM1 and CMTM2. Although mouse *Cmtm1* is a homolog of human *CMTM1*, mouse *Cmtm2a* and *Cmtm2b* are two homologs of human *CMTM2*. The reference URL is indicated as follows; http://www.treefam.org/family/TF338711#tabview=tab1.
(B), (C) Box indicates a match in all sequences. Two-way arrows indicate putative transmembrane regions. In mice and humans, there are four transmembrane regions in CMTM1 (B), CMTM2A (C), and CMTM2B (C).









D

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wt(1-133 AAs)     :MNPASARRESGGDGSRRESRRLTLRPRVLDASSPSDSQAYAASAPPHQGP
                    ||||||||||||||||
mutant(1-133 AAs):MNPASARRESGGDGSRLYDLECWMLPHLRIHRRMLPLLHLTRDLQYPGHQ

wt(1-133 AAs)     :SVPGTPASQGPSVPGTPASQGPSVPGTPASQGPSVPGTPASQGPSVPGTP
mutant(1-133 AAs):PARDLQYPGHQPARDLQYPGHQPARDLQYPGHQPARDLQYPEHHPASNLL

wt(1-133 AAs)     :SRQQSFIRATPIRQQSFIRAIPSRQRPSVQAIP
mutant(1-133 AAs):YVPHQSASNLLYVLYHPGSDLQYKPYHPASDL*
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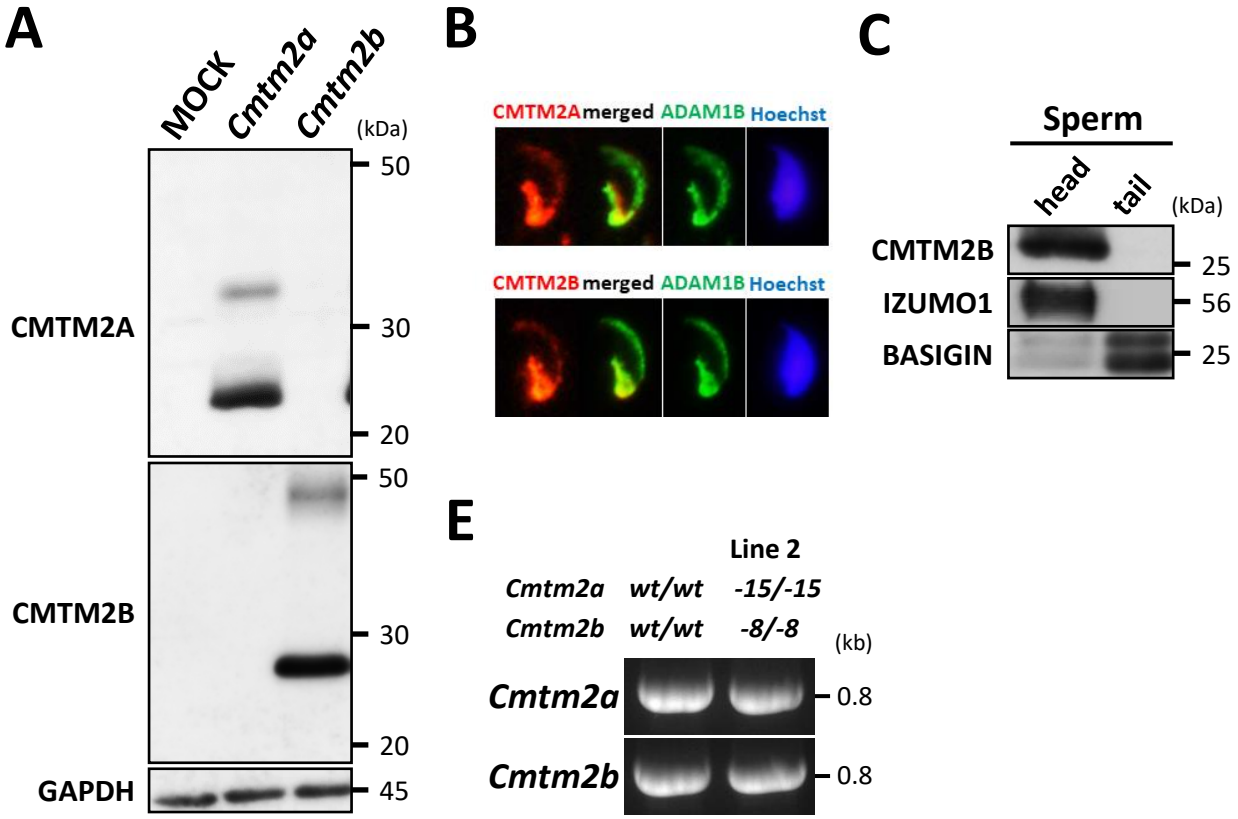
Figure S2. Generation of *Cmtm1* mutant mice with CRISPR/Cas9.
(A) Waveforms of direct sequencing from genomic DNA in *Cmtm1* mutant mice.
(B) RT-PCR using testis cDNAs in *Cmtm1* mutant mice. The PCR product in wild-type testes is represented by 1176-bp.
(C) Waveforms of direct sequencing from testis cDNA in *Cmtm1* mutant mice.
(D) Amino acid sequence of *Cmtm1* mutant mice. The 19 bp deletion caused a frameshift mutation leading to a premature termination codon after the amino acid 132th.
(E) Representative testicular histology sections stained with hematoxylin and eosin. Spermatogenesis in *Cmtm1*$^{-19/-19}$ mice looked normal compared with that of wild-type mice. Scale bars: 200 µm.



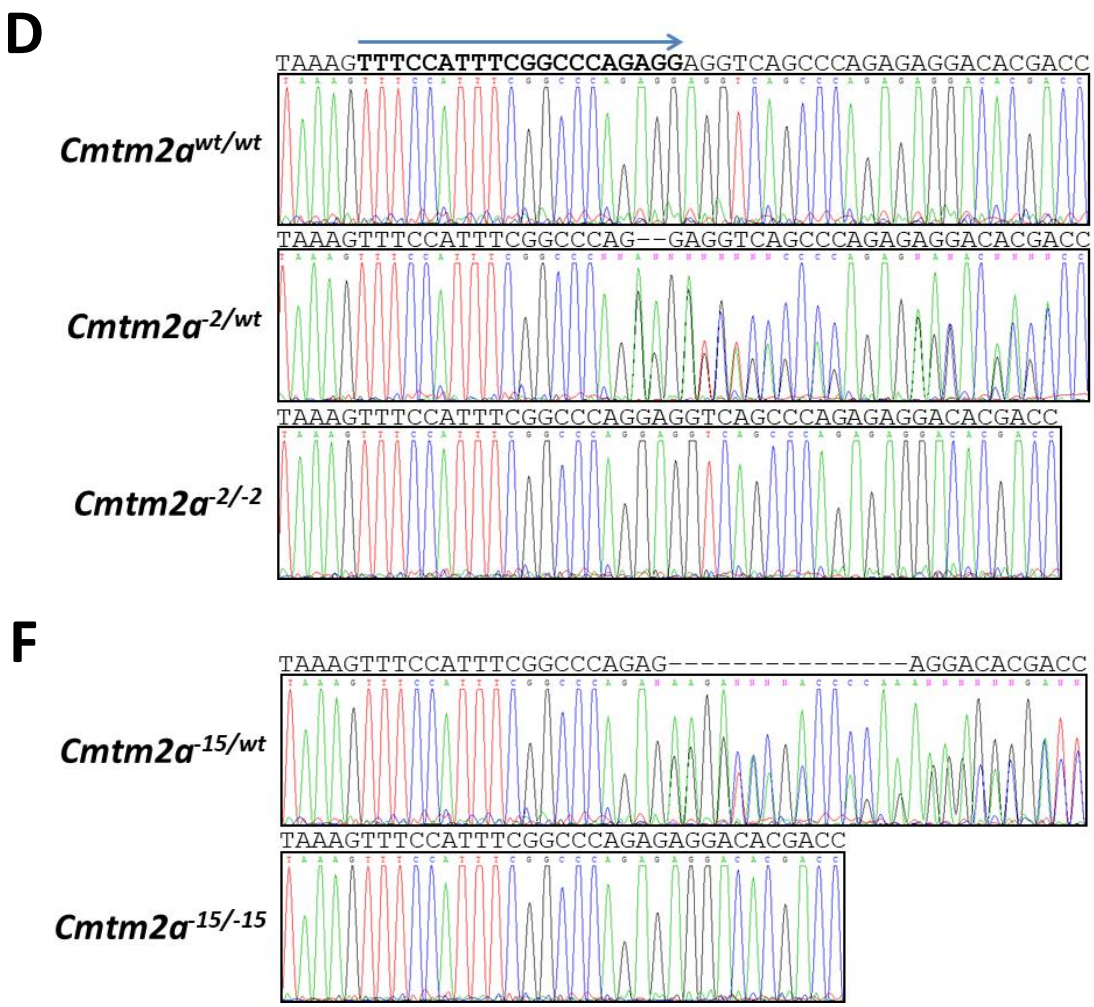


Figure S3. Immunoblot of CMTM2A/2B and generation of *Cmtm2a* and *Cmtm2b* double mutant mice with CRISPR/Cas9.

(A) Immunoblot of CMTM2A and CMTM2B using transfected HEK293T cells. Expression plasmids of mouse *Cmtm2a* and *Cmtm2b* were introduced using full-length cDNA cloned into a pCAG1.1 vector containing the *CAG* promoter and a rabbit globin poly(A) signal. MOCK represents pCAG1.1 empty vector.

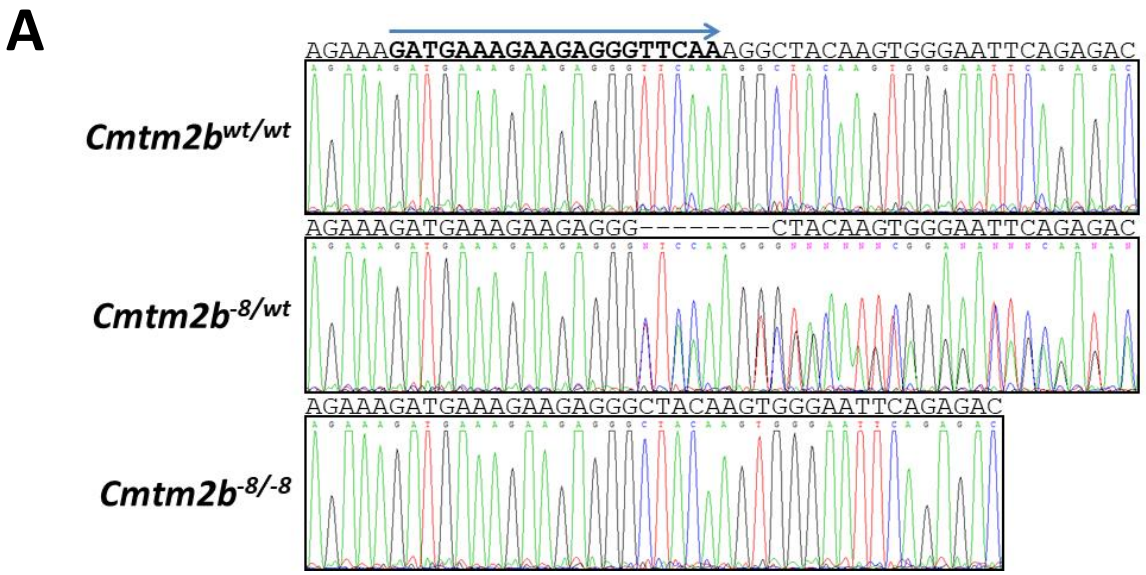
(B) Immunostaining of CMTM2A (upper), CMTM2B (lower), and ADAM1B in cauda epididymal spermatozoa. CMTM2A and CMTM2B, red signals, localized to the plasma membrane on the sperm head. ADAM1B, green signals, was also used as a marker for the plasma membrane of sperm head. Blue signals indicate sperm nuclei stained with Hoechst 33342.

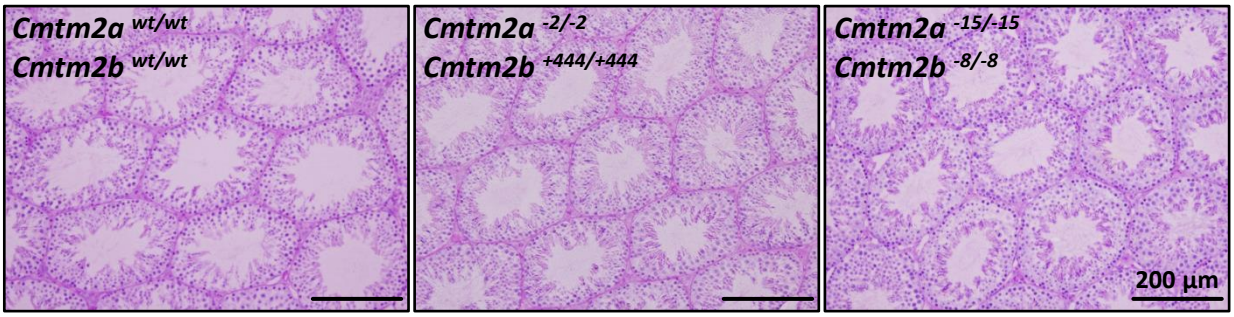
(C) Immunoblot of CMTM2B using sperm lysates collected after sperm head-tail separation.

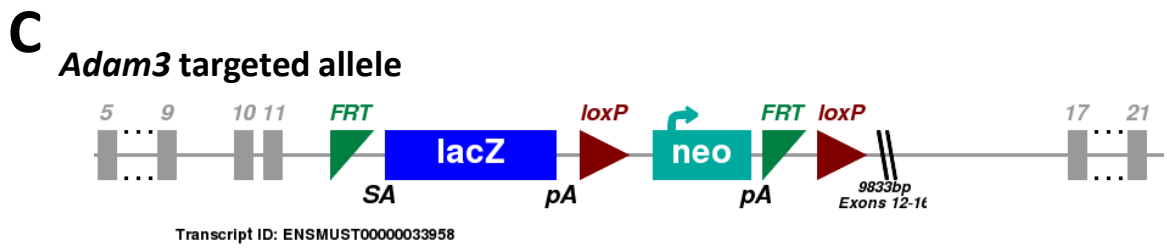
(D) Waveforms of direct sequencing of the 2 bp deletion (5'-AG-3') in *Cmtm2a* gene.

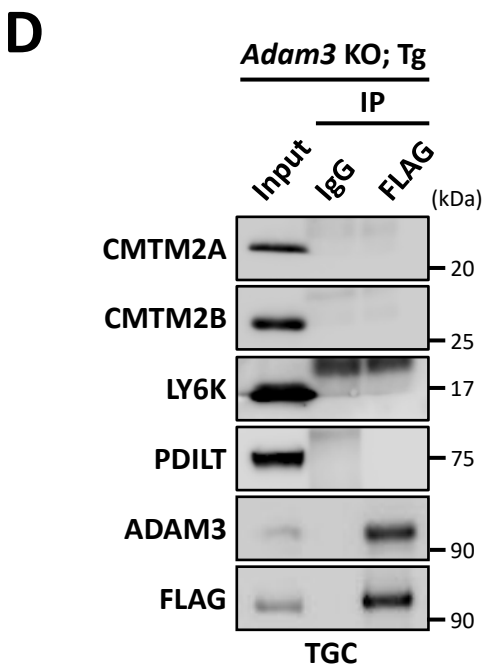
(E) Genotyping with PCR in *Cmtm2a*$^{-15/-15}$; *2b*$^{-8/-8}$ (Line 2) mice.

(F) Waveforms of direct sequencing of the 15 bp deletion (5'-GAGGTCAGCCCAGAG-3') in *Cmtm2a* gene.









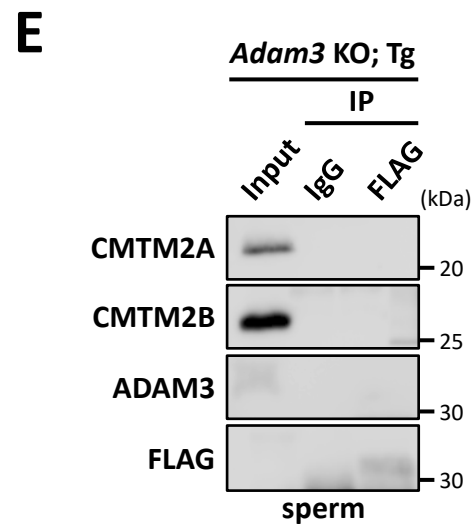


Figure S4. Analysis of *Cmtm2a/2b* double mutant mice and *Adam3-Flag* Tg rescued *Adam3* KO mice.

(A) Waveforms of direct sequencing of 8 bp deletion (5'-TTCAAAGG-3') in *Cmtm2b* gene.

(B) Representative testicular histology sections stained with hematoxylin and eosin. Spermatogenesis in *Cmtm2a*$^{-2/-2}$; *2b*$^{+444/+444}$ (Line 1), and *Cmtm2a*$^{-15/-15}$; *2b*$^{-8/-8}$ (Line 2) mice looked normal compared with that in wild-type mice. Scale bars: 200 µm.

(C) Image of targeted disruption of *Adam3* gene. *Adam3* exons 12-16 are replaced with an IRES:lacZ trapping cassette and a floxed promoter-driven neo cassette. The reference URL indicates as follows;
https://www.mousephenotype.org/data/genes/MGI:102518.

(D) Immunoprecipitation analysis of TGC lysates using the FLAG antibody.

(E) Immunoprecipitation analysis of *Adam3-Flag* Tg sperm lysates using the FLAG antibody.

Table S1. Sequence information of *Cmtm2a/2b* mutant mice

Genotype	Sequence (5' to 3')	Name
+444 bp of *Cmtm2b* allele	TTCAAAGGCTACAAGTGGGAATTCAGAGACAGCAATAAAGA CTTCTGGGCGCAGGGACATGCAGAGTGCAAGTCTCTGATTAT GGTAAACTAGATTGGAATGACAGAGGTGTTGGTGCGGGTGG GGGTGGGGTTCCTCAAAAGCAGGGTAGAGCCCTTGCAACCCC TCCGGTGTACGTATATTTTTCCATTCAGATCCTCCTCATTGCT GCAATGGTCTGTTTCCAAAGAGTGGCCACTCATCCAATTGTA ATCCTGCTGCTTACTATGGAACTGTCAATCTGTGCTTTCTTCT TCTTCCTCTACTCCTTAGC	313 bp deletion
	CCTTCTAGATTTAAATAACTTTAAAAGTCCCACAGTCTTTACA TATTCTTAAAATTTCAATTTCTTTAAAATAGCCATCTCTTTTA AAATCCAAAGTCTTTTTACAATTAAAAGTCTCTTAACTGTGG GCTCCACTAAAACAGTTTCTTCCTTCGAGAGGGAAAATATCA GGGCACAGCCACAATCAAAAGCAAAAGTCAATCTCCAACCG TCCAATGTCTGGGATCCAACTTACAATCTTCTGGGCTCCTCCA AGGGCTTGGGTCACTTCTCCAGCCATGCCCTTTGTAGCACAC GCGTCATCCTCTAGGCTCCAGATGCCTGTACTCCACTGCTGCT GCTGCTCTTGGTGGTCATCTCATGGTACTGGCATCTCCAAAAC ACTGCATGACCCCTTCAGTCCTGGGCCATCAATTGCAACTGA GGCTGCACTTTCACCAATGGCCTTCCATGGCCTCTCACAGTGC CAAGCCTCAGCTGCTCTGCTCAACTCCTTCATGCCTTCAAAAC CAGTACCACCTGGGTGACCCTTACACATTACCAAGTCCAGCC ACAGCACAAGGTACAACTTTGGCTATATCTGGAACACAGCCA CTGTGCTTTCAGAAAATACTTCCCAGAAGATGTCACCTCAAT GATGCTGGTCTCTTCTTAATCACCGCTAATTTCTTAGCTCCAG CTAACCAGCATCAATAGTCCCAGTAATGCAAAGTTTTTGCTTT AGTAGTTCTGGTATCTTGTTAATCACAGCTGATGG	757 bp insertion

Table S2. Sequence information of *Adam3-Flag* Tg mice

Genotype	Sequence (5' to 3')	Name
2208 bp of modified mouse *Adam3* cDNA	ATGCTGCCCTTATTCCTAGTCCTCTCATACCTAGGCCAAGTGATCGCT GCAGGCAAAGACGTAGAAACACCACTTCTGCAGATCACAGTACCAGA GAAAATCGACACCAACATCCAGGATGCCAAGGAGGCAGAGACACAA GTCACTTACGTGGTCAGAATTGAGGGCAAAGCATACACACTCCAACT TGAAAAACAGTCATTTTTACACCCACTTTTTGGAACATATTTGCGTGA CAAGTTGGGAACATTGCAACCATACTTTTCTTTAGTAAAGACTCACTG CTTTTATCAAGGACATGCTGCCGAGATCCCAGTATCAACAGTGACACT CAGCACTTGCTCAGGTCTCAGGGGTTTGTTGCAGTTAGAGAACATCAC TTATGGAATTGAGCCCTTGGAGTCTTCAGCTACATTTGAACACATACT TTATGAAATTAAGAATAACAAAATTGACTATTCTCCTTTAAAAGAAA ACTTCGCAAATTCTGAGCAAGAAAGTCAGTCCTACCGAATCCTTGTCA AACCAGAGAAAGGTTCGAACTCGACACTAACAAAGAGAATTCTGAG GATAAAGATCATCATGGATAAAGCCATGTTTGACCATATGGGCTCTG AGGTGGGAGTTGCAACTCAGAAAGTTGTTCACATCTTTGGTCTCATTA ATACTATGTTTTCCCAGCTGAAGATGACAGTGATGTTAAACTCTCTGG AGATCTGGTCAGAACAAGACAAGATAGAAACCAATGGGGATGCTGA TGAGGTCCTGCAAAGGTTCCTGCTGTGGAAAAGCAAGGAAATATCTC AAAAGGCCCAGGATATAACCTACTTACTCCTGTATAAGGATCATCCT GATTATGTGGGAGCGACATATCATGGGATGGCCTGCAACCCAAATTT TACCGCAGGAATTGCTCTGCATCCAAAGACGTTAGCTGTGGAGGGCT TTGCCATTGTTCTCTCACAGTTGCTGGGAATTAACCTGGGGTTGGCAT ACGATGACGTCTACAATTGTTTCTGTCCAGGAAGCACATGCATAATG AATCCTTCGGCAATACGTTCCCAAGGCATAAAGGTTTTTAGCAGTTGT AGTGTGGATGAATTCAAACAGCTGGCTTCACAACCTGAACTGGACTG CCTTCGGAATACATCGGAGACAGAATTTGTTGTCCAGCCCCAAGGTG GATCGTATTGTGGTAACCATCTCCTGGAGGTCCCAGAGCAATGTGACT GTGGCCCACCAGAGACCTGTACTCATAAAAAATGCTGTAACCCTAAA GACTGTACTCTGATTGATGCTGCGCAGTGTGGTACAGGGCCATGCTGT GATAAAAGAACATGTACGATAGCCGAAAGAGGACGCCTATGCAGAA AAAGTAAGGACCAGTGTGATTTCCCAGAGTTCTGCAATGGAGAAACT GAAGGCTGTGCCCCTGATACAAAAGCTGCAGACCTGGAACCTTGCAA CAACGAGACTGCCTATTGCTTTGGTGGGGTTTGCCGAGACCCAGATA GGCAGTGTACAGATTTATTTGGGAAATATGCTAAGGGTCCTAATTATG TGTGTGCACAAGAAGTGAATCTGCAAAATGACAAATTTGGAAACTGT CATGGGCGATGTAATTATAGTGCTATATTTTGTGGAAAAGCAGTTTGC TACTGGAACTTTGCAGAAGTCATACAAACAGAGAAATATGATGTTCA GTATACTTACTTGGGAGGCCAGGTGTGTGTGTCCGCTCATTTAAGATC ACAGACTGGGACTAGAGATGACACATATGTCCATGATGGCACTGTTT GTGGTAGTGGCCAGGTTTGCTTCCGAGGAGATTGCTTACGTGTGCATG TCCTAAGGGGAACCAGGGAGTGTGAGGCCGATGACAAATGCCAAGG ACACGGGATTTGCAACAATCTAAACAACTGTCAGTGTGAATCTGGTTT TGCTCCTCCAGAGTGTGACATGACACCTTCATCTCCAGGAGGAAGCA TGGATGACGGATTTTGGCTGCCCTTCGATAAAAGCACACCTTTGATTT TCAAGCGGCATGGCCTTAAGTACAAAAAAGTGCTTTTGATCAGTTTCT ATATCCTCCTGCCTTTCCTTGTTGTGCTAGCCTTCATGGCTGTTAAGCG GATGATTGGTAAGCGGCTTGCTAAGCAGAATATCTCAAAGGCATTGG AGCACAAAGAGGAAGCATTCAACAGAGGG	*Adam3*-Flag transgene

Table S3. List of primers

Figure	Sequence (5' to 3')	Name
1A, 1B, S2B-C	ATGAATCCTGCATCCGCCAGGC	*Cmtm1*
	TCAGTGCTGCTTCTGCCTCAGTTG	
	ATGGCAGCACCGATAAAGTTTCCATTTCG	*Cmtm2a*
	TTACCACTTCCTTAACCTAAGGCCTCTG	
	GATCTGATGAACGACCTGGCCTGTTC	*Cmtm2b*
	TCACCATCCAGGGTCCATCGAC	
	ATGTGGCCCCCGGACGC	*Cmtm3*
	TTAGTCAGAGTCTGAGTCCGAGTTGGAG	
	GAGTGCTCCCCGTGCGAAGG	*Cmtm4*
	TCATGTGTCCAGGCGCTGGATC	
	ATGGAGACTCCACGGCCGGTC	*Cklf*
	TTATTCTCTGTCATCGATGTCATTAGTAGACTTTTTCTG	
	AAGTGTGACGTTGACATCCG	*Actb*
	GATCCACATCTGCTGGAAGG	
2C, S3D-F	CTGTCTGGTTCCTGTTGCATGGAGG	*Cmtm2a* genotyping
	CGAGGACAGCAAAGCCTTTGCGTC	
	GCATGGAGGAGATCCTGCTGGTCC	*Cmtm2b* genotyping
	GGACAGCAAAGCCTTTGGTTCTTCTCTC	
2D, S3A	ATGGCAGCACCGATAAAGTTTCCATTTCG	*Cmtm2a* cDNA
	TTACCACTTCCTTAACCTAAGGCCTCTG	
	ATGGCAGCGCCCGCGC	*Cmtm2b* cDNA
	TCACCATCCAGGGTCCATCGAC	
4C	ACAGCTCACTCCATTGCATTTGCC	*Adam3* genotyping
	CTGGTTAGCACACAAGCAAAGGGG	
	ATCCGGGGGTACCGCGTCGAG	
	TTGAGCGGGCCGCTTGCGCACTGG	Tg genotyping
	CCTGAGCAAGTGCTGAGTGTCAC	
S2A	GCATGTGGTTGCTGGGTCTTGAG	*Cmtm1* genotyping
	CTGTCGGCAATGCGATCTCTGTACAC	
S4C	ATCCGGGGGTACCGCGTCGAG	*Adam3* 3'-arm screening
	GCAAGAATAGCATCGACCACACACTAGAG	
Table. S2	TCTAGAGCCGCCATGCTGCCCTTATTCCTAGTCCTCTCATAC	*Adam3-Flag* transgene
	GATATCCCCTCTGTTGAATGCTTCCTCTTTGTG	